CHAPTER 5

SUMMARY

It is now well established that rat spermatidal protein TP2, which is a small basic nuclear protein of 114 residues, has DNA condensing properties It is also a zinc metalloprotein with two atoms of zinc coordinated per molecule involving cysteine and histidine residues It has been shown that this zinc induced structure of TP2, is crucial for its preference towards GC rich sequences for DNA condensation and also for the recognition of human CpG island sequence

Rat TP2 has a number of cysteine and histidine residues in the N-terminal two-thirds of the protein (Figure 17), many more than that required for coordinating two zinc atoms The lack of sequence similarity between the zinc binding domain of TP2 and other zinc finger proteins, made it very challenging to identify the actual residues involved in zinc coordination. It is very crucial to identify the exact cysteine and histidine residues that coordinate with zinc in order to understand the 3-dimensional structure of this zinc metalloprotein Such information would also be very valuable in interpreting the DNA binding properties of TP2. The present investigation was initiated to identify specific cysteine and histidine residues involved in zinc coordination, to understand a) the zinc induced fold polypeptide structure of TP2, b) and the roles of its zinc binding domain in DNA condensation Results obtained from the present investigation are summarized below

1. Rat TP2 cDNA was cloned by the RT-PCR method and its nucleotide sequence was determined. The sequence of the first 24 nucleotides (extreme 5' end) before the proline codon CCC was found to be completely different from that reported earlier but was found to match completely with the genomic sequence deposited in the EMBL databank.

2 A low level of expression was achieved when TP2 was expressed in *E coli* using the expression vector, pTrc99A The recombinant TP2 was purified using Ni^{2+}-agarose and heparin-agarose chromatography and was shown to be authentic by Western blotting, radioactive ^{65}Zinc-blotting and N-terminal protein sequencing analysis

3 Since the expression level in the pTrc99A background was very low, an attempt was made to increase the level of expression by both codon optimization and engineering the vector encoded 5' UTR A five-fold increase in the expression level of recombinant TP2 was thus obtained in the T7 driven expression vector, pET-22b (+)

4 Serendipitously it was observed that plasmid encoded β-lactamase was induced by TP2 in BL21 (DE3) cells This hyperinduction of β-lactamase appears to be unique to TP2 as the same phenomenon was not observed when H1d, another basic protein, was overexpressed in the same vector

5 A method for the generation of multiple site-specific mutations in a single PCR product was developed This method, wherein a single PCR product carries all required mutations, has proved valuable in our present study as it precludes the repeated clonings of PCR products bearing single mutations, phosphorylation of primers, other primer annealing specifications and also the requirement of thermostable DNA ligase

6. To identify the zinc coordinating amino acids, the above mentioned protocol was used to generate a range of 40 single and multiple site-specific mutants spanning the entire array of Histidine and Cysteine residues (His to Gln and Cys to Ala) in the zinc binding region of TP2 and their zinc binding activity was determined using radioactive ^{65}Zn-blotting

7 ^{65}Zn-blotting studies of all the mutants of TP2 have distinctly shown the presence of two zinc binding pockets The multiple TP2 mutant bearing all histidine to glutamine mutations showed only 55-60 % zinc binding activity while the multiple cysteine

mutants retained 55-60 % zinc binding activity Further fine mapping with various mutants (single and a combination of multiple mutants) has revealed that the first pocket involves all histidine residues, H12, H14, H16 and H24, while C29, C31, C35 and C38 are involved in the second zinc finger

8 The zinc binding domain of TP2 possesses many unique features involvement of only histidine residues in the first finger, lack of the conserved hydrophobic amino acid residues in the zinc finger, and unique spacing of amino acid residues between the zinc coordinating ligands of each finger

9 Two proline residues bridge the zinc coordinating ligands in the first finger Although prolines are implicated in bending of polypeptides, our mutational studies have shown no specific contribution of the two prolines towards zinc binding

10 *In vitro* DNA condensation of both poly(dG-dC) poly(dG-dC) and rat oligonucleosomal DNA by various zinc mutants of TP2 were studied using Circular Dichroism Spectroscopy Mutation in either one or both the zinc binding pockets resulted in a loss of DNA condensation to the same extent in both poly(dG-dC) poly(dG-dC) and rat oligonucleosomal DNA, even though the overall condensation of poly(dG-dC).poly(dG-dC) by TP2 was found to be higher than that of rat oligonucleosomal DNA as expected This suggests that the simultaneous binding of both the zinc fingers is required for DNA condensation

11. The presence of two lone arginine residues at position 20 and 36 in the third loop of the two zinc fingers respectively (Figure 38), prompted us to investigate their role in DNA condensation Circular Dichroism Spectroscopic analysis of R20A, R36A and R20,36A mutants of TP2 revealed that the arginine residues in the two fingers were found to be absolutely essential for DNA condensation even though their zinc binding ability was not compromised. Similar to the zinc finger mutants mentioned above, the simultaneous interaction of both the arginine residues with DNA was found to be required to bring about appreciable DNA condensation